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# Retardation of Cochlear Maturation and Impaired Hair Cell Function Caused by Deletion of All Known Thyroid Hormone Receptors

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The deafness caused by early onset hypothyroidism indicates that thyroid hormone is essential for the development of hearing. We investigated the underlying roles of the TR $\alpha$ 1 and TR $\beta$  thyroid hormone receptors in the auditory system using receptor-deficient mice. TR $\alpha$ 1 and TR $\beta$ , which act as hormone-activated transcription factors, are encoded by the *Thra* and *Thrb* genes, respectively, and both are expressed in the developing cochlea. TR $\beta$  is required for hearing because TR $\beta$ -deficient (*Thrb*<sup>tm1/tm1</sup>) mice have a defective auditory-evoked brainstem response and retarded expression of a potassium current ( $I_{K,f}$ ) in the cochlear inner hair cells. Here, we show that although TR $\alpha$ 1 is individually dispensable, TR $\alpha$ 1 and TR $\beta$  synergistically control an extended array of functions in postnatal cochlear development. Compared with *Thrb*<sup>tm1/tm1</sup> mice, the

deletion of all TRs in *Thra*<sup>tm1/tm1</sup>*Thrb*<sup>tm1/tm1</sup> mice produces exacerbated and novel phenotypes, including delayed differentiation of the sensory epithelium, malformation of the tectorial membrane, impairment of electromechanical transduction in outer hair cells, and a low endocochlear potential. The induction of  $I_{K,f}$  in inner hair cells was not markedly more retarded than in *Thrb*<sup>tm1/tm1</sup> mice, suggesting that this feature of hair cell maturation is primarily TR $\beta$ -dependent. These results indicate that distinct pathways mediated by TR $\beta$  alone or by TR $\beta$  and TR $\alpha$ 1 together facilitate control over an extended range of functions during the maturation of the cochlea.

**Key words:** cochlea; development; tectorial membrane; hair cell; thyroid hormone receptor; transcription factor

Thyroid hormone is essential for the development of hearing. Deafness arises if there is insufficient hormone available during sensitive periods in the fetal and possibly early neonatal period in humans (Trotter, 1960; Morreale de Escobar et al., 1996) and in the neonatal period in rodents (Deol, 1973; Van Middlesworth and Norris, 1980; Uziel, 1986). Despite the well known requirement for thyroid hormone, however, relatively little is known of the receptor pathways underlying the actions of this hormone in the auditory system.

The TR $\alpha$ 1 and TR $\beta$  thyroid hormone receptors, encoded by the related *Thra* and *Thrb* genes, respectively, act as hormone-activated transcription factors (Sap et al., 1986; Weinberger et al., 1986), and both are expressed in the developing cochlea. The *Thrb* gene is expressed in the organ of Corti, which contains the sensory hair cells, where it is prominently expressed in the greater

epithelial ridge. *Thra* is more widely expressed throughout the cochlea (Bradley et al., 1994; Lauterman and ten Cate, 1997; Knipper et al., 1998). The expression patterns of TR $\alpha$ 1 and TR $\beta$  suggest that the cochlea is a direct site of action of thyroid hormone, consistent with findings of morphological abnormalities in the organ of Corti in hypothyroid rodents (Deol, 1973, 1976; Uziel et al., 1981; O'Malley et al., 1995). Hypothyroidism retards the development of the greater epithelial ridge and malforms the tectorial membrane (TM), which normally contacts the stereociliary bundles of the mechanosensitive hair cells. An intact TM is necessary for the response of the hair cell to acoustic stimulation and for the tuning of basilar membrane motion mediated by the electromotility of the outer hair cells (Dallos et al., 1996; Legan et al., 2000; Steel and Kros, 2001). Although thyroid hormone is known to be required for the morphological differentiation of the organ of Corti, less is known of the role of this hormone in the development of the physiological functions of the cochlea.

We showed previously that TR $\beta$ -deficient (*Thrb*<sup>tm1/tm1</sup>) but not TR $\alpha$ 1-deficient (*Thra*<sup>tm1/tm1</sup>) mice have impaired auditory-evoked brainstem responses (Forrest et al., 1996; Rüscher et al., 1998). The deletion of TR $\beta$  also results in deafness in a human kindred with recessive resistance to thyroid hormone (Refetoff et al., 1967), and mild hearing loss has been reported in 20% of the dominant cases of this syndrome that are associated with TR $\beta$  point mutations (Brucker-Davis et al., 1996). *Thrb*<sup>tm1/tm1</sup> mice have developmentally retarded expression of a potassium current,  $I_{K,f}$ , in their inner hair cells (Rüscher et al., 1998).  $I_{K,f}$  normally

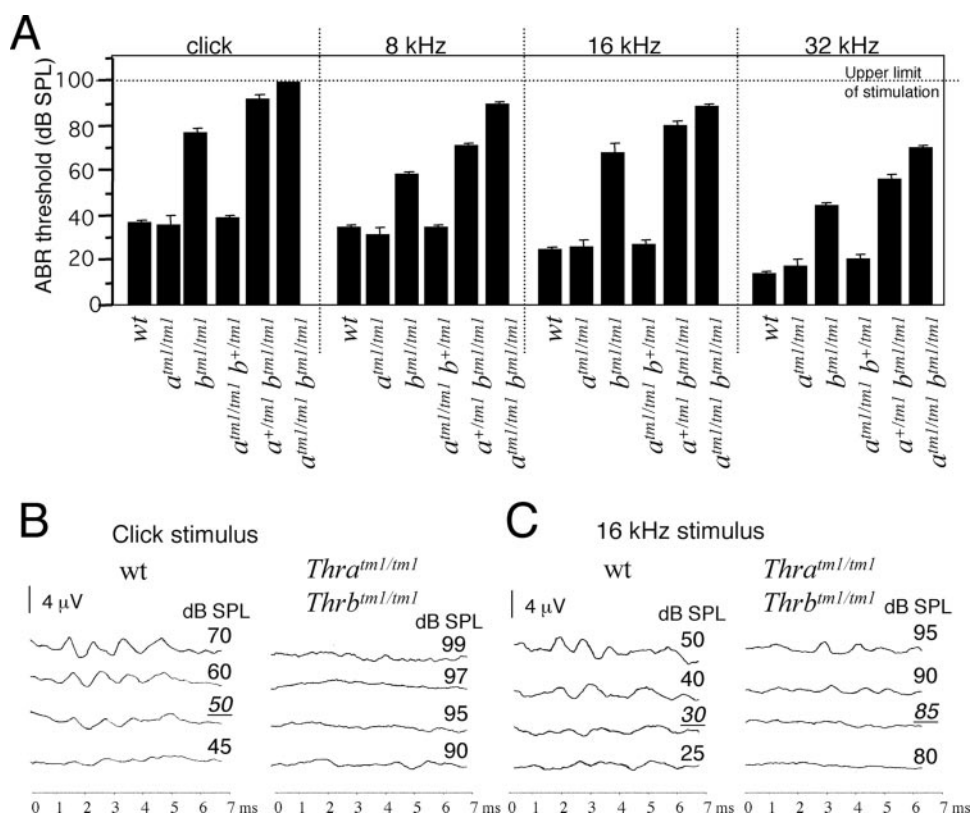
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**Figure 1.** ABR thresholds in mice with single or combined deletions of TR $\alpha$ 1 and TR $\beta$ . *A*, Mean ABR thresholds  $\pm$  SEM (in decibels of SPL) for wild-type (wt),  $Thra^{tm1/tm1}$  ( $\alpha^{tm1/tm1}$ ),  $Thrb^{tm1/tm1}$  ( $\beta^{tm1/tm1}$ ),  $Thra^{tm1/tm1}Thrb^{tm1/tm1}$  ( $\alpha^{tm1/tm1}\beta^{tm1/tm1}$ ) mice, or other combined mutant strains. All genotypes were on a uniform, congenic C57BL/6J ( $N > 10$ ) background. Responses to click, 8, 16, and 32 kHz stimuli are shown. Groups shown contained  $n = 7$ –8 mice at 5–13 weeks of age. Thresholds were undetectable or were significantly elevated ( $p < 0.01$ ) in  $Thra^{tm1/tm1}Thrb^{tm1/tm1}$  mice compared with wt or  $Thrb^{tm1/tm1}$  mice for all stimulus frequencies tested. *B*, *C*, Representative ABR waveforms for wt and  $Thra^{tm1/tm1}Thrb^{tm1/tm1}$  mice in response to click (*B*) and 16 kHz (*C*) stimuli. Waveforms are shown on a 4  $\mu$ V fixed scale for comparison (actual thresholds were determined on a normalized scale for sensitivity; see Materials and Methods). Thresholds are underlined. No waveform was detectable for  $Thra^{tm1/tm1}Thrb^{tm1/tm1}$  mice for the click stimulus. Only weak, atypical waveforms were detected for the 16 kHz stimulus shown.

becomes active with the onset of auditory function at approximately postnatal day 13 (P13) and is thought to transform the immature hair cell into a high-frequency signal transmitter (Kros et al., 1998).  $Thrb^{tm1/tm1}$  adult mice however, do not display major hypothyroid-like cochlear malformations (Forrest et al., 1996). Therefore, to investigate interactions between  $Thra$  and  $Thrb$  in cochlear development, we generated  $Thra^{tm1/tm1}Thrb^{tm1/tm1}$  mice that lack all known TRs (Göthe et al., 1999). The results reveal that TR $\alpha$ 1 and TR $\beta$  together facilitate control over an extended and novel array of functions in cochlear maturation.

## MATERIALS AND METHODS

**Mouse strains.** The  $Thrb^{tm1Df}$  targeted mutation deletes all known  $Thrb$  products (Forrest et al., 1996). The  $Thra^{tm1Ven}$  mutation specifically deletes the TR $\alpha$ 1 receptor product of  $Thra$  while leaving intact the TR $\alpha$ 2 nonreceptor splice variant product of this gene (Wikström et al., 1998). Some histology and physiological studies were performed on mutant mice with a mixed background of equal parts 129/Sv, C57BL/6J, 129OlaHsd, and BALB/c strains. To remove background variability for auditory-evoked brainstem response (ABR) measurements,  $Thrb^{tm1}$  and  $Thra^{tm1}$  mutations were separately backcrossed for 12 and 9 generations, respectively, to create congenic C57BL/6J strains. These strains were intercrossed to generate  $Thra^{tm1/tm1}Thrb^{tm1/tm1}$  mice on a C57BL/6J background; wild-type (wt) C57BL/6J mice do not show hearing loss until  $>6$  months of age (Zheng et al., 1999). ABR results were similar on the mixed background except that variation was more marked.  $Thra^{+tm1}Thrb^{+tm1}$  or  $Thra^{tm1/tm1}Thrb^{+tm1}$  mice were interbred to generate  $Thra^{tm1/tm1}Thrb^{tm1/tm1}$  mice; double mutants themselves displayed infertility (Göthe et al., 1999). Genotypes were determined by PCR as described (Forrest et al., 1996; Wikström et al., 1998). Animal experiments followed all applicable guidelines and approved institutional protocols at Mount Sinai School of Medicine, the Karolinska Institute, and the University of Tübingen.

**ABR.** ABR tests were performed with a SmartEP ABR system, version 2.1, from Intelligent Hearing Systems (Miami, FL) essentially as described (Zheng et al., 1999). Mice were anesthetized with avertin (0.25 mg/gm body weight) and active, reference, and ground electrode needles were placed subcutaneously at the vertex, ventrolateral to the left ear,

and ventrolateral to the right ear, respectively. Binaural stimulation was presented with a rise-fall time of 1.5 msec, at a rate of 25/sec. ABR thresholds were determined using decreasing intervals of 10 dB sound pressure level (SPL), which were reduced to 5 dB SPL determine the lowest threshold with visually recognizable ABR peaks on a normalized scale. Results were comparable with previous recordings with an older version of the apparatus from Intelligent Hearing Systems (Forrest et al., 1996). Slightly lower thresholds were detected at 32 kHz in the present work, possibly because of the elimination of background strain variability or minor differences in the physical set up of the machines. Both machines were calibrated by the manufacturer. The ABR was analyzed on two series of mice on the congenic background. In addition to the results shown in Figure 1*A*, a second series of  $n = 52$  mice (males and females at ages of 5–12 weeks), including an additional  $n = 7$   $Thra^{tm1/tm1}Thrb^{tm1/tm1}$  mice, gave comparable results.

**Whole-cell recording.** The recording technique has been reported previously (Kros et al., 1998; Rüsch et al., 1998). Briefly, hair cells were studied after acute dissection of the most apical half-turn of the organ of Corti from acute at different postnatal ages. The isolated piece of the organ of Corti was mounted in a chamber and perfused at 10 ml/hr with an extracellular solution composed of (in mM): 144 NaCl, 0.7 NaH<sub>2</sub>PO<sub>4</sub>, 5.8 KCl, 1.3 CaCl<sub>2</sub>, 0.9 MgCl<sub>2</sub>, 5.6 D-glucose, and 10 HEPES-NaOH, pH 7.3. Vitamins and amino acids for Eagle's minimal essential medium were added from concentrate (Life Technologies).

Membrane currents and voltages were studied at room temperature (20–25°C) by whole-cell patch-clamp using an Axopatch 200B amplifier. Patch pipettes were filled with an intracellular solution proven to sustain Ca<sup>2+</sup> currents in hair cells (Platzter et al., 2000) (in mM): 135 KCl, 0.1 CaCl<sub>2</sub>, 3.5 MgCl<sub>2</sub>, 5 K<sub>2</sub>EGTA, 2.5 Na<sub>2</sub>ATP, and 5 HEPES-KOH, pH 7.3. Currents under voltage clamp are presented with capacitive transient and linear leak currents subtracted; all voltages were corrected for the voltage drop across the uncompensated series resistance. Voltages were also corrected for the liquid junction potential between the intracellular and extracellular solutions (−4 mV; as calculated by computer software by Peter Barry, University of New South Wales, Australia). Fifteen inner hair cells (IHCs) of the most apical half-turn of the cochlea from  $Thra^{tm1/tm1}Thrb^{tm1/tm1}$  mice had a mean resting membrane potential of  $-69 \pm 7$  mV, which was similar to the mean  $-70 \pm 8$  mV measured in 12  $Thra^{tm1/tm1}Thrb^{+tm1}$  and  $Thra^{tm1/tm1}$  mice. The IHC fast current  $I_{K,f}$  was measured at −25 mV between 2.4 and 3.6 msec after the onset of the



depolarizing voltage step, as shown in Figure 4A. The fits of the developmental expression pattern are according to a sigmoidal logistic growth curve:

$$I = (I_{\max} - I_{\min}) / (1 + \exp(-s(t - t_{1/2}))) + I_{\min},$$

where  $I$  is current (in nanoamperes),  $s$  is a slope factor ( $d^{-1}$ ),  $t$  is time (measured in days), and  $t_{1/2}$  is the time at which  $I$  is halfway between  $I_{\max}$  and  $I_{\min}$ .

**Capacitance.** Motility-related nonlinear capacitance was measured as described previously (Oliver and Fakler, 1999). Briefly, outer hair cells (OHCs) were whole-cell voltage clamped, and their membrane capacitance was monitored using a software lock-in technique while the voltage was ramped from  $-120$  to  $+50$  mV. Capacitance was plotted as a function of membrane potential and fitted with the derivative of a Boltzmann function (Santos-Sacchi, 1991). Values of nonlinear capacitance are given relative to the linear membrane capacitance of the cell (in femtofarads per picofarad) as determined from current transients induced by  $10$  mV voltage steps.

**Endocochlear potentials.** Procedures followed described methods (Steel and Barkway, 1989; Rüsch et al., 1998). Mice were anesthetized with 20% urethane at  $0.01$  ml/g body weight. For  $Thra^{tm1/tm1}Thrb^{tm1/tm1}$  mice, the surgical procedure had to be modified because of their 15-fold enlarged thyroid gland (Göthe et al., 1999). A tracheotomy was not performed, and the head was held at the dorsal skull using dental cement. The cochlea was exposed as described previously (Rüsch et al., 1998). Potential measurements used a custom-made amplifier as a high impedance voltmeter. Statistical tests were two-tailed Student's  $t$  tests.

**Histology, cell counts, and electron microscopy.** Mice were killed with  $CO_2$ , then the temporal bones were isolated rapidly and fixed in 3% glutaraldehyde/2% paraformaldehyde in PBS by overnight immersion.  $Thra^{tm1/tm1}Thrb^{tm1/tm1}$  cochlear samples were decalcified in  $0.2$  M EDTA in PBS for 7–21 d, then embedded in methacrylate (Immunobed; Polysciences, Warrington, PA) and sectioned at  $3$ – $5$   $\mu$ m on a rotary microtome for histology; sections were stained with thionin. Cochleas from  $n \geq 3$  mice per genotype at a given age were studied. Other cochleas were embedded in OCT glue and cryosectioned at  $7$ – $10$   $\mu$ m for immunostaining. Antibodies against  $\alpha$ - and  $\beta$ -tectorins were used at  $1:1000$  dilution, and specific fluorescent staining was detected using FITC-conjugated secondary antibodies. Staining against otogelin was performed as described (Legan et al., 2000).

For cell counts, after fixation, the bony labyrinth, scala vestibuli, and Reissner's membrane were dissected to expose the apical surface of the sensory epithelium. The sensory epithelium was cut into thirds and placed on glass slides as whole mounts. Total numbers of hair cells were counted for  $\geq 3$  cochleas per genotype for P8 pups and for adults using differential interference contrast microscopy.

For transmission electron microscopy, cochleas were rapidly removed, placed in PBS, the oval and round windows were removed, and a small hole was made in the apex of the bony capsule. Fixative (2.5% glutaraldehyde in  $0.1$  M sodium cacodylate, pH 7.2, containing 1% tannic acid) was gently perfused through the opened windows, and the apical hole and cochleas were then immersed in the same fixative for a further 2 hr. Tissue pieces were washed three times in  $0.1$  M sodium cacodylate buffer, pH 7.2, post-fixed in 1% osmium tetroxide in  $0.1$  M sodium cacodylate, pH 7.2, washed with cacodylate buffer, and decalcified in  $0.5$  M EDTA for 10–14 d at  $4^\circ C$ . After decalcification, tissues were dehydrated through a series of ascending concentrations of ethanol, equilibrated with propylene oxide, and imbedded in TAAB 812 resin (TAAB Laboratories Equipment Ltd., Reading, UK). Blocks were cured for 2 d at  $60^\circ C$ . For light microscopy of  $Thrb^{tm1/tm1}$  mice,  $1$ – $\mu$ m-thick sections were cut with glass knives and stained with Toluidine blue.

Ultrathin sections were cut with a diamond knife, double stained with uranyl acetate and lead citrate, and viewed with a Hitachi 7100 electron microscope operating at  $75$  kV. Cochleas from a total of 10 1- to 7-month-old wild-type mice, seven 4- to 6-month-old  $Thra^{tm1/tm1}$   $Thrb^{tm1/tm1}$  mice, and six 1- to 4-month-old  $Thrb^{tm1/tm1}$  mice were examined.

## RESULTS

### Combined roles of TR $\beta$ and TR $\alpha$ 1 in auditory function

The auditory-evoked brainstem response, an overall measure of auditory function, is impaired in TR $\beta$ -deficient  $Thrb^{tm1/tm1}$  mice but is normal in TR $\alpha$ 1-deficient  $Thra^{tm1/tm1}$  mice (Forrest et al.,

1996; Rüsch et al., 1998). To investigate interactions between the *Thra* and *Thrb* genes, we analyzed the ABR in  $Thra^{tm1/tm1}Thrb^{tm1/tm1}$  mice lacking all known TRs (Fig. 1).  $Thra^{tm1/tm1}Thrb^{tm1/tm1}$  mice were 30% smaller than wt mice but were viable, and despite fertility problems, thrived reasonably well (Göthe et al., 1999), thus allowing study of auditory function. ABR thresholds were assessed on a uniform, congenic C57BL/6J background to preclude hearing loss because of background strain variations.

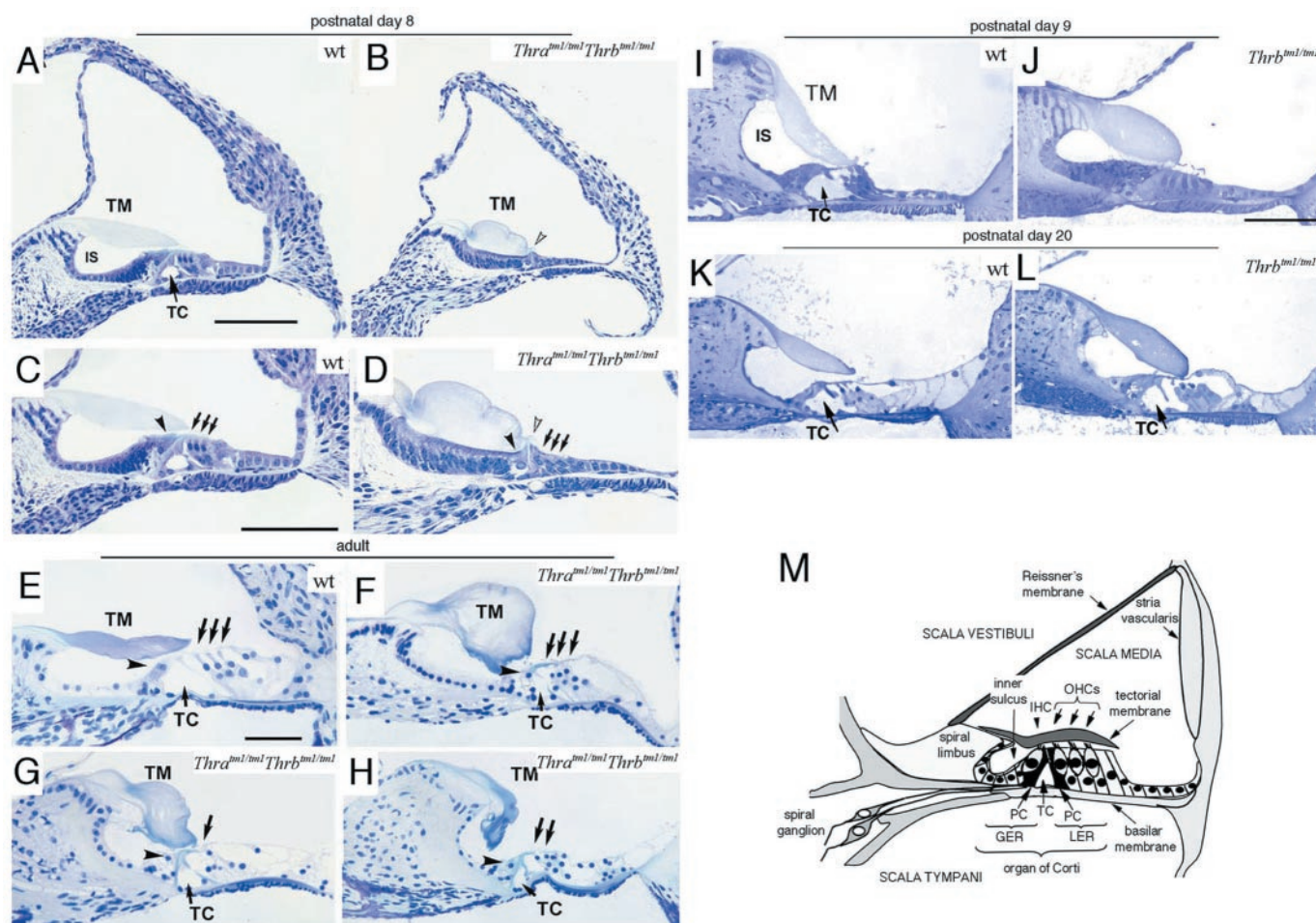
$Thra^{tm1/tm1}Thrb^{tm1/tm1}$  mice (shown as  $a^{tm1/tm1}b^{tm1/tm1}$  in Fig. 1A) had significantly exacerbated defects compared with  $Thrb^{tm1/tm1}$  mice (shown as  $b^{tm1/tm1}$ ) ( $p < 0.01$ ) for click and pure tone stimuli (8, 16, 32 kHz) that span the sensitive hearing range of mice.  $Thra^{tm1/tm1}Thrb^{tm1/tm1}$  mice lacked any detectable click response at the upper limit of stimulation of the testing apparatus (100 dB SPL) and showed only weak, atypical responses for high-frequency stimuli (16 and 32 kHz). The waveforms of the residual responses to high frequencies could only be evoked with much elevated stimulus intensities (85 dB SPL threshold for 16 kHz shown in Fig. 1C) and were abnormal because the initial peaks within the first 2–3 msec of stimulation that are usually the most prominent in mice (Zheng et al., 1999) were absent or delayed.  $Thra^{+/tm1}Thrb^{tm1/tm1}$  mice had intermediate thresholds between those of  $Thrb^{tm1/tm1}$  and  $Thra^{tm1/tm1}Thrb^{tm1/tm1}$  mice, indicating a dosage function for TR $\alpha$ 1.  $Thra^{tm1/tm1}Thrb^{+/tm1}$  mice, however, had normal thresholds, indicating that a single *Thrb*<sup>+</sup> wild-type allele was sufficient to sustain auditory function. The results demonstrate major interactions between *Thra* and *Thrb* in the auditory system.

### Cochlear morphology in TR-deficient mice

Morphological abnormalities were detected in the cochlea in  $Thra^{tm1/tm1}Thrb^{tm1/tm1}$  mice that indicated a role for TRs during the postnatal differentiation of the greater epithelial ridge of the organ of Corti (Fig. 2). During the first postnatal week, the TM, an extracellular matrix (Richardson et al., 1987), is secreted and extends from the spiral limbus across the inner sulcus to contact the hair cells in the sensory epithelium (Fig. 2A,C and see schematic diagram in Fig. 2M). The inner sulcus opens in association with the retraction of the epithelial cell layer underlying the TM. In  $Thra^{tm1/tm1}Thrb^{tm1/tm1}$  pups at P8, the TM was enlarged and deformed and remained attached to the underlying epithelium; moreover, no inner sulcus was formed (Fig. 2B,D). The tunnel of Corti between the inner and outer pillar cells had not opened, and the pillar cells formed a pyramidal rise above the surface of the sensory epithelium (Fig. 2D).

In adult  $Thra^{tm1/tm1}Thrb^{tm1/tm1}$  mice, the inner sulcus had opened below the TM and the sensory epithelium was differentiated (Fig. 2E–H). These results suggested that TRs are not required for the subsequent development of the greater epithelial ridge but rather that they determine the correct timing of the developmental progression. The TM however, remained permanently malformed in adults, and it was enlarged in apical and mid-turns of the cochlea (Fig. 2F,G), whereas it was often retracted in basal turns (Fig. 2H). An occasional but minimal loss of hair cells was observed in some adult but not P8 cochleas (Fig. 2G,H). Systematic counts, however, showed that the hair cell loss was not statistically significant (Table 1). No obvious defects were observed in other regions of the cochlea.

Although TR $\beta$ -deficient mice lack gross malformations in the cochlea as adults (Forrest et al., 1996), these mice showed some delay in the earlier postnatal development of the organ of Corti that represents a milder form of the phenotype present in



**Figure 2.** Cochlear malformations in *Thra*<sup>tm1/tm1</sup>*Thrb*<sup>tm1/tm1</sup> (A–H) and *Thrb*<sup>tm1/tm1</sup> (I–L) mouse strains. A–H, *Thra*<sup>tm1/tm1</sup>*Thrb*<sup>tm1/tm1</sup> and wt control mice at postnatal day 8 (A–D) and as adults (7- to 8-week-old) (E–H). A, C, Low (A) and higher (C) magnification view of a mid-modiolar, midbasal turn of the cochlea from a wt pup at P8 showing the tectorial membrane (TM) extending over the inner sulcus (IS) to the hair cells (the TM is slightly retracted from the IS because of shrinkage during fixation). The tunnel of Corti (TC) has opened between the inner and outer pillar cells. The filled arrowhead indicates an IHC, and the three arrows indicate OHCs. Abbreviations and symbols are the same in other panels; see M, for full description. B, D, Low (B) and higher (D) magnification view of a midbasal turn from a *Thra*<sup>tm1/tm1</sup>*Thrb*<sup>tm1/tm1</sup> pup. The TM is enlarged, the IS has not formed, the tunnel of Corti has not opened, and the pillar cells protrude (open arrowhead) above the epithelium between the IHC and OHCs. The greater epithelial ridge below the TM is markedly thicker than in the wt pup. Scale bars: A (same for B), C (same for D), 100  $\mu$ m. E, Apical turn of the cochlea from an adult wild-type mouse. F, G, H, Apical (F), mid (G), and basal (H) turns of the cochlea of a *Thra*<sup>tm1/tm1</sup>*Thrb*<sup>tm1/tm1</sup> adult. The tunnel of Corti and IS have opened. An occasional but minimal loss of hair cells was evident in mid- and basal turns. The TC is present but appears somewhat misshapen. The TM is enlarged and deformed, and in basal turns is often shriveled and retracted into the IS. Scale bar: E (same in F–H), 50  $\mu$ m. I–L, Cochlear basal turn from *Thrb*<sup>tm1/tm1</sup> and wt control mice at P9 (I, J) and at P20 (K, L). J, In *Thrb*<sup>tm1/tm1</sup> pups at P9, the formation of the IS is retarded, the TM is slightly enlarged, the underlying epithelial cell layer is thicker than in wt controls, and the tunnel of Corti (TC) is unopened. K, In wt mice at P20, the organ of Corti has matured (the TM in this example is slightly lifted above the OHCs because of shrinkage during fixation). L, In *Thrb*<sup>tm1/tm1</sup> mice at P20, the IS has opened, but the TM is slightly enlarged; the epithelium lining the IS is slightly thicker than in wt controls. The *Thrb*<sup>tm1/tm1</sup> phenotype is milder than in *Thra*<sup>tm1/tm1</sup>*Thrb*<sup>tm1/tm1</sup> mice. I–L represent 1  $\mu$ m sections stained with toluidine blue (and thus differ from the 3  $\mu$ m, thionin-stained sections in A–H). Cochleas from  $n \geq 3$  mice per genotype per age were examined. M, Schematic diagram of major structures of the cochlea. The hair cells reside on the basilar membrane and lie below the tectorial membrane. IHC, Inner hair cell; OHC, outer hair cell; PC, pillar cell; TC, tunnel of Corti; GER, greater epithelial ridge; LER, lesser epithelial ridge of the organ of Corti.

*Thra*<sup>tm1/tm1</sup>*Thrb*<sup>tm1/tm1</sup> mice (Fig. 2I–L). The formation of the inner sulcus was delayed, and the tunnel of Corti remained unopened at P9 in *Thrb*<sup>tm1/tm1</sup> mice (Fig. 2J). The TM also showed some enlargement, although not to the extent occurring in *Thra*<sup>tm1/tm1</sup>*Thrb*<sup>tm1/tm1</sup> mice. In wt weanlings at P20, the organ of Corti had matured (Fig. 2K), and normal ABR thresholds could be recorded (Forrest et al., 1996). In *Thrb*<sup>tm1/tm1</sup> mice at P20, however, the TM was slightly enlarged, although it did extend to the hair cells and was not grossly misshapen as in *Thra*<sup>tm1/tm1</sup>*Thrb*<sup>tm1/tm1</sup> mice. Thus, TR $\beta$  has an individual role in the timely differentiation of the organ of Corti and the correct

formation of the TM. However, major control of these processes is conferred by TR $\alpha$ 1 and TR $\beta$  acting together.

The TM also exhibited ultrastructural disarray in *Thra*<sup>tm1/tm1</sup>*Thrb*<sup>tm1/tm1</sup> mice (Fig. 3A,B). Transmission electron micrographs showed that the major collagenous fibrils were present but that the striated sheet matrix (Hasko and Richardson, 1987) was disorganized. Thus, TR $\beta$  and TR $\alpha$ 1 jointly exert major control over both the ultrastructure and the overall form of the TM. The loss of TR $\beta$  alone resulted in a subtle form of this phenotype. Although in adult *Thrb*<sup>tm1/tm1</sup> mice, the TM was not grossly malformed (Forrest et al., 1996), the organization of the striated

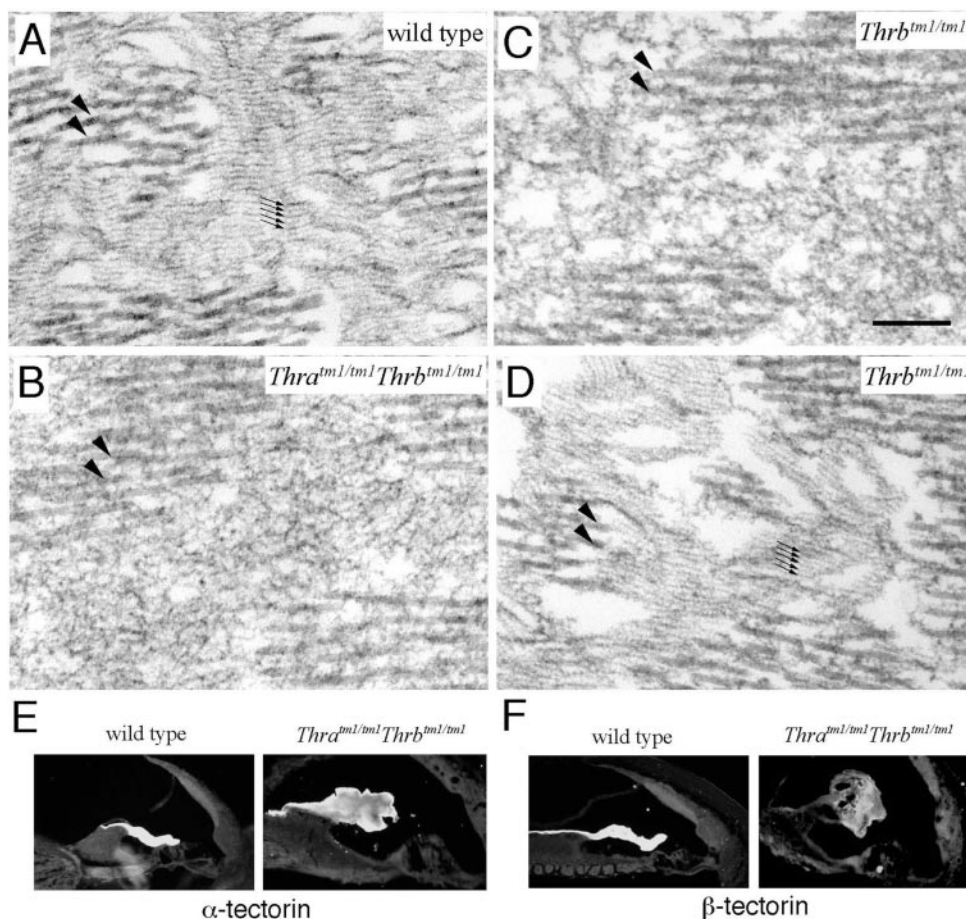


**Table 1.** Hair cell densities<sup>a</sup> in adult wild-type and *Thra*<sup>tm1/tm1</sup>*Thrb*<sup>tm1/tm1</sup> mice

Genotype	Inner hair cell density	Outer hair cell density	Total hair cell density
Wild type	12.7 ± 0.4	36.3 ± 0.8	48.9 ± 1.1
<i>Thra</i> <sup>tm1/tm1</sup> <i>Thrb</i> <sup>tm1/tm1</sup>	12.5 ± 0.2	34.3 ± 1.0	46.7 ± 0.8

<sup>a</sup>Mean densities ± SEM per 100 μm length of the inner hair cell row in the basal-apical axis of the organ of Corti. Basal, mid-, and apical regions of the cochlea were counted, then results were pooled to determine overall averages. There was no statistically significant difference between mutant and wild-type mice (Student's *t* test). *n* = 3 mice/genotype.

**Figure 3.** Tectorial membrane malformation in *Thra*<sup>tm1/tm1</sup>*Thrb*<sup>tm1/tm1</sup> and *Thrb*<sup>tm1/tm1</sup> mice. *A, B*, Transmission electron micrographs showing the ultrastructure of the matrix in the region of the TM that overlies the sensory hair cells in the organ of Corti in adult wt (*A*) and *Thra*<sup>tm1/tm1</sup>*Thrb*<sup>tm1/tm1</sup> (*B*) mice. In wt mice, the major 20 nm diameter collagen fibrils (two arrowheads) are seen embedded in a striated sheet matrix formed from alternating light and dark staining, fine diameter filaments (five small arrows). In the *Thra*<sup>tm1/tm1</sup>*Thrb*<sup>tm1/tm1</sup> mouse, collagen fibrils are present, but the striated sheet matrix is disorganized throughout the TM. *C, D*, Partial disarray of TM ultrastructure in *Thrb*<sup>tm1/tm1</sup> mice. The TM displays a similar disarray as *Thra*<sup>tm1/tm1</sup>*Thrb*<sup>tm1/tm1</sup> mice in the upper region (*C*) (i.e., those regions that are located furthest from the apical surface of the organ of Corti). The lower region (*D*) appears normal. Micrographs shown are from a 7-month-old wt mouse (*A*), a 6-month-old *Thra*<sup>tm1/tm1</sup>*Thrb*<sup>tm1/tm1</sup> mouse (*B*), and a 4-month-old *Thrb*<sup>tm1/tm1</sup> mouse (*C, D*). Scale bar: *C* (same in *A, B, D*), 200 nm. *E, F*, Representative immunostaining for α-tectorin (*E*) and β-tectorin (*F*) in cochlear sections of adult wt and *Thra*<sup>tm1/tm1</sup>*Thrb*<sup>tm1/tm1</sup> mice. In *Thra*<sup>tm1/tm1</sup>*Thrb*<sup>tm1/tm1</sup> mice, the malformation of the TM is evident, but it is still immunoreactive for the tectorins. Similar results were found for otogelin (data not shown).



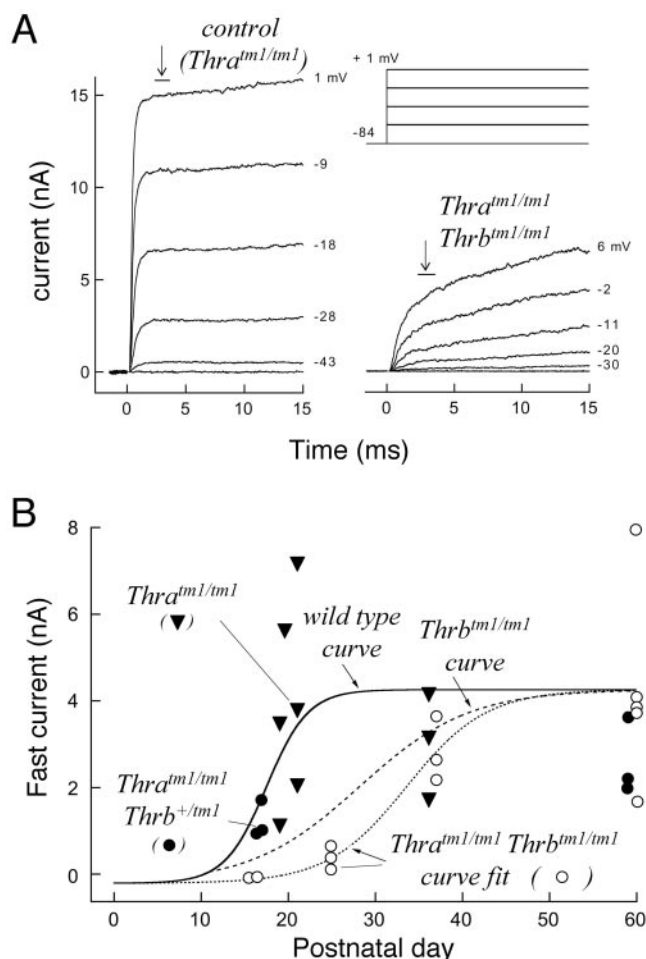
sheet matrix was disrupted in some areas. The disorganization was limited to upper regions of the TM (Fig. 3C), whereas lower regions appeared normal (Fig. 3D). Staining with antibodies against α- and β-tectorins (Legan et al., 2000) and otogelin (Simmler et al., 2000) in cochlear sections from *Thra*<sup>tm1/tm1</sup>*Thrb*<sup>tm1/tm1</sup> mice yielded positive signals that confirmed the TM deformities but did not show any gross absence of these major components (Fig. 3E,F).

### Cochlear physiology in TR-deficient mice

We investigated a role for TRs in the physiological differentiation of hair cells because mouse hair cells mature during the early postnatal period when thyroid hormone is required for the development of hearing. With the onset of auditory function at approximately P13, IHCs express the fast-activating potassium current  $I_{K,f}$  that is associated with maturation of the IHC (Kros et al., 1998). Normally,  $I_{K,f}$  expression begins by P13 and plateaus after P20 (Fig. 4B, wild-type curve), whereas we have shown that  $I_{K,f}$  induction is retarded in *Thrb*<sup>tm1/tm1</sup> mice (Fig. 4B,

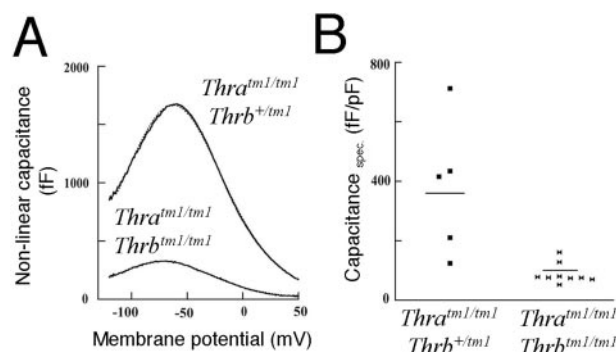
*Thrb*<sup>tm1/tm1</sup> curve) (Rüsch et al., 1998). The induction of  $I_{K,f}$  was found to be similarly retarded in *Thra*<sup>tm1/tm1</sup>*Thrb*<sup>tm1/tm1</sup> mice. Figure 4A shows examples of the voltage-activated currents of a control IHC from a *Thra*<sup>tm1/tm1</sup> mouse at P21, which activated rapidly to steady state levels within 2 msec at potentials between −43 and +1 mV, whereas the fast current was absent in a *Thra*<sup>tm1/tm1</sup>*Thrb*<sup>tm1/tm1</sup> mouse at P25. Analysis of IHCs (*n* = 13) from *Thra*<sup>tm1/tm1</sup>*Thrb*<sup>tm1/tm1</sup> mice over a range of postnatal ages showed that  $I_{K,f}$  was eventually expressed and followed a logistic growth function that reached half-maximal expression at a time point ( $t_{1/2}$ ) of 33.6 d with a slope factor  $s$  = 0.22/d (Fig. 4B, *Thra*<sup>tm1/tm1</sup>*Thrb*<sup>tm1/tm1</sup> curve fit), compared with the wt curve, where  $t_{1/2}$  = 17.5 d and  $s$  = 0.42/d (see Materials and Methods) (Rüsch et al., 1998).  $I_{K,f}$  values of IHCs (*n* = 15) from normal-hearing *Thra*<sup>tm1/tm1</sup> or *Thra*<sup>tm1/tm1</sup>*Thrb*<sup>+/tm1</sup> control mice closely followed the previously described developmental profile of wt mice (Kros et al., 1998; Rüsch et al., 1998) (Fig. 4B).

The similar pattern of retardation in *Thra*<sup>tm1/tm1</sup>*Thrb*<sup>tm1/tm1</sup>



**Figure 4.** Delayed expression of the  $I_{K,f}$  fast current in inner hair cells of  $Thra^{tm1/tm1}Thrb^{tm1/tm1}$  mice. **A**, Fast-activating currents ( $I_{K,f}$ ) in IHCs of a  $Thra^{tm1/tm1}$  mouse at P21 and a  $Thra^{tm1/tm1}Thrb^{tm1/tm1}$  mouse at P25. The fast component  $I_{K,f}$  was largely missing in the  $Thra^{tm1/tm1}Thrb^{tm1/tm1}$  IHC. Arrows and bars indicate the time window during which the amplitude of  $I_{K,f}$  was measured at  $-25$  mV. The voltage step protocol used is indicated above the traces of the  $Thra^{tm1/tm1}Thrb^{tm1/tm1}$  cell. **B**, Developmental profile of  $I_{K,f}$  at  $-25$  mV in IHCs of  $Thra^{tm1/tm1}Thrb^{tm1/tm1}$  mice. Currents were measured as indicated by the arrow and bar in **A**, and they were plotted versus the depolarizing membrane potential to obtain  $I-V$  plots.  $I_{K,f}$  was then measured at  $-25$  mV from these plots by interpolating data points negative and positive to  $-25$  mV and plotted versus the postnatal age of the mouse. The solid line is the previously determined curve for  $I_{K,f}$  in IHCs of wt mice and the broken (dashed) line the curve for  $Thrb^{tm1/tm1}$  mice as reported (Rüsch et al., 1998). The dotted line is the logistic growth function fitted to the data points plotted for IHCs of  $Thra^{tm1/tm1}Thrb^{tm1/tm1}$  mice (circular points), where the current maximum and minimum were fixed to the values of the wt fit [ $4.28$  nA and  $-208$  pA ( $-0.208$  pA)];  $t_{1/2} = 33.6$  d and slope =  $0.22/d$ . Data points for  $I_{K,f}$  from IHCs of normal-hearing  $Thra^{tm1/tm1}Thrb^{+/+}$  mice (filled circles), and  $Thra^{tm1/tm1}$  littermates (inverted triangles) followed the normal wt developmental profile.

and in  $Thrb^{tm1/tm1}$  mice indicated that  $I_{K,f}$  induction is primarily dependent on TR $\beta$ . The eventual rise of  $I_{K,f}$  in IHCs of  $Thra^{tm1/tm1}Thrb^{tm1/tm1}$  mice however, shows that none of the known TRs are ultimately necessary for  $I_{K,f}$  induction and indicates rather that TRs confer correct timing over  $I_{K,f}$  expression during IHC maturation. IHCs of  $Thra^{tm1/tm1}Thrb^{tm1/tm1}$  mice at stages when  $I_{K,f}$  was absent did develop small currents with slow kinetics that did not reach steady state during the 15 msec time interval shown in Figure 4A. This type of current resembled the  $I_{K,s}$



**Figure 5.** Reduced nonlinear capacitance in outer hair cells in  $Thra^{tm1/tm1}Thrb^{tm1/tm1}$  mice. **A**, Measurements of nonlinear capacitance in a  $Thra^{tm1/tm1}Thrb^{tm1/tm1}$  mouse and a control ( $Thra^{tm1/tm1}Thrb^{+/+tm1}$ ) mouse. **B**, Plot of values of the nonlinear capacitance in five OHCs of three  $Thra^{tm1/tm1}Thrb^{+/+tm1}$  control mice and in 10 OHCs of three  $Thra^{tm1/tm1}Thrb^{tm1/tm1}$  mice at P8. The capacitance was significantly smaller in OHCs from  $Thra^{tm1/tm1}Thrb^{tm1/tm1}$  mice ( $p < 0.001$ ).

slow current component described previously for IHCs of wt mice (Kros et al., 1998), suggesting that despite the lack of  $I_{K,f}$  IHCs in  $Thra^{tm1/tm1}Thrb^{tm1/tm1}$  mice retained other functional properties of IHCs.

We also investigated whether TRs control the acquisition of the physiological properties of the OHCs, the second cochlear hair cell type. Most prominently, OHCs display a unique electromotility that is believed to facilitate the active amplification process of the cochlea (Holley, 1996). The underlying electromechanical transduction mechanism is based on conformational changes of a voltage-sensitive membrane motor protein, recently identified as prestin (Zheng et al., 2000). We assessed electromechanical transduction by measuring the voltage-dependent capacitance prestin imposes on the OHC membrane (Santos-Sacchi, 1991; Zheng et al., 2000) (Fig. 5). OHCs from  $Thra^{tm1/tm1}Thrb^{tm1/tm1}$  pups at P8 had a significantly reduced ( $p < 0.001$ ) nonlinear capacitance (mean  $\pm$  SD;  $87 \pm 32$  fF/pF) compared with the normal values of  $Thra^{tm1/tm1}Thrb^{+/+tm1}$  littermates ( $379 \pm 228$  fF/pF) or the values described previously for wt pups at P8 ( $290 \pm 47$  fF/pF) (Rüsch et al., 1998). OHCs of  $Thrb^{tm1/tm1}$  pups at P8 were previously reported to have slightly reduced nonlinear capacitance ( $150 \pm 46$  fF/pF) (Rüsch et al., 1998). Thus, TRs are important for the acquisition of OHC electromotile properties. The slight impairment in  $Thrb^{tm1/tm1}$  pups suggests that TR $\beta$  has a specific role. However, the more marked defect in  $Thra^{tm1/tm1}Thrb^{tm1/tm1}$  pups suggests that this function is largely coregulated by both TR $\alpha 1$  and TR $\beta$ .

The potassium-rich endolymph of the scala media (see Fig. 2M) is normally maintained at a high positive resting potential that is necessary for auditory function. This endocochlear potential (EP) contributes to the driving force for mechanoelectrical transduction by the hair cells (Rübsamen and Lippe, 1997). The EP was reduced in adult  $Thra^{tm1/tm1}Thrb^{tm1/tm1}$  mice ( $52.3 \pm 13.5$  mV;  $p < 0.001$ ) compared with the normal values in  $Thra^{tm1/tm1}Thrb^{+/+tm1}$  mice ( $100.3 \pm 9.0$  mV) or the values shown previously in wt and  $Thrb^{tm1/tm1}$  mice (Table 2) (Steel and Barkway, 1989; Rüsch et al., 1998). The occurrence of the low EP only in  $Thra^{tm1/tm1}Thrb^{tm1/tm1}$  mice lacking all TRs suggests that the EP is normally coregulated by both TR $\alpha 1$  and TR $\beta$  and that these receptors are functionally interchangeable in developing the ability to generate the EP.

**Table 2. Endocochlear potentials of TR-deficient mouse strains**

	Wild type <sup>a</sup>	<i>Thrb</i> <sup>tm1/tm1</sup>	<i>Thra</i> <sup>tm1/tm1</sup> <i>Thrb</i> <sup>+/tm1</sup>	<i>Thra</i> <sup>tm1/tm1</sup> <i>Thrb</i> <sup>tm1/tm1</sup>
Endocochlear potential (mV)	92.5 ± 13.5	85.2 ± 13.5	100.3 ± 9.0	52.1 ± 13.6*
<i>n</i> =	4	6	4	4 <sup>b</sup>

<sup>a</sup>Values for wild-type and *Thrb*<sup>tm1/tm1</sup> mice are shown for comparison and are taken from Rüsch et al. (1998).

<sup>b</sup>*Thra*<sup>tm1/tm1</sup>*Thrb*<sup>tm1/tm1</sup> mice were prone to die under anesthesia, and six additional mice died during attempted recording of endocochlear potentials.

\**p* < 0.001 compared with *Thra*<sup>tm1/tm1</sup>*Thrb*<sup>+/tm1</sup> or wild-type mice.

**Table 3. Summary of auditory system phenotypes in mice lacking TRα1 (*Thra*<sup>tm1/tm1</sup>), TRβ (*Thrb*<sup>tm1/tm1</sup>), or both TRα1 and TRβ (*Thra*<sup>tm1/tm1</sup>*Thrb*<sup>tm1/tm1</sup>)**

Phenotype	Severity in TR-deficient mouse strains		
	<i>Thra</i> <sup>tm1/tm1</sup>	<i>Thrb</i> <sup>tm1/tm1</sup>	<i>Thra</i> <sup>tm1/tm1</sup> <i>Thrb</i> <sup>tm1/tm1</sup>
ABR impaired	–	++	+++
Tectorial membrane deformed	ND	+	+++
Inner sulcus, delayed differentiation	ND	+	++
IHC, <i>I<sub>K,f</sub></i> expression retarded	–	++	++
OHC, nonlinear capacitance impaired	–	+	++
Endocochlear potential reduced	–	–	+

(–), No phenotype detected; (+), (++) or (+++) indicate presence of phenotype and approximate severity (+++, most severe); ND, not determined; IHC, inner hair cell; OHC, outer hair cell.

## DISCUSSION

The phenotype of *Thra*<sup>tm1/tm1</sup>*Thrb*<sup>tm1/tm1</sup> mice unmasks a role for TRα1 that was not evident in mice lacking only TRα1 and also indicates that TRα1 and TRβ together control novel cochlear functions. These common TR functions include a major role in the formation of the TM and in the development of the endocochlear potential and the electromechanical transduction properties of outer hair cells (Table 3). The masking of the full extent of these phenotypes in the single receptor gene deletions suggests a functional overlap between TRα1 and TRβ consistent with their related transactivation properties on several different DNA response elements *in vitro* (Jeannin et al., 1998; Wahlstrom et al., 1999). These results therefore suggest that the variety of actions provided by two related receptor genes extends the range of functions that may be controlled by thyroid hormone in cochlear development.

The unique role of TRβ, evident in the somewhat less severe phenotype in *Thrb*<sup>tm1/tm1</sup> mice, may reflect differences in receptor expression levels in specific cochlear cell types (Bradley et al., 1994) such that inadequate levels of TRα1 fail to substitute for the loss of TRβ. It is also possible that structural distinctions between TRα1 and TRβ partly constrain the ability of TRα1 to regulate a critical subset of TRβ target genes in the cochlea. TRα1 and TRβ diverge completely in the N terminus, which plays a role in DNA binding stability and in the transactivation properties of the receptor. They also have certain differences in their central DNA binding domains which contribute to functional differences on some response elements *in vitro* (Lezoualc'h et al., 1992; Sjöberg and Vennström, 1995; Zhu et al., 1997). The identification of the direct, downstream target genes that mediate the physiological actions of TRs in the cochlea may allow the elucidation of the basis of this TR isotype-specificity.

The cochlear abnormalities in *Thra*<sup>tm1/tm1</sup>*Thrb*<sup>tm1/tm1</sup> mice that lack all known TRs resemble the defects described in hypothyroid mice or rats, which suggests that TRα1 and TRβ together

account for the known functions of thyroid hormone in the cochlea. The induction of hypothyroidism in mice and rats during a critical, early window beginning at or before the time of birth (Deol, 1973; Uziel et al., 1981; Uziel, 1986) causes a similar retardation in the formation of the inner sulcus and deformity of the TM as is found in *Thra*<sup>tm1/tm1</sup>*Thrb*<sup>tm1/tm1</sup> mice. Our findings thus argue against the hypothetical existence of any other unknown TRs or non-TR-mediated mechanism of action of thyroid hormone in cochlear development. A range of cloning and functional studies suggest that TRα1 and TRβ represent the full complement of nuclear TRs (Gauthier et al., 1999; Göthe et al., 1999). However, the growth retardation and other phenotypes of *Thra*<sup>tm1/tm1</sup>*Thrb*<sup>tm1/tm1</sup> mice are somewhat milder than the phenotypes of severe hypothyroidism, a distinction that has raised the possibility of non-TR pathways of action of thyroid hormone or of hormone-independent actions of TRs in some systems (Göthe et al., 1999). The similar cochlear phenotypes of hormone- or TR-deficient mice, however, make it unlikely that such mechanisms need be invoked in the cochlea. Although current evidence allows us to draw a conclusion regarding cochlear morphology, these comparisons cannot be extended to the physiological defects we report for *Thra*<sup>tm1/tm1</sup>*Thrb*<sup>tm1/tm1</sup> mice, because cochlear physiology has been little studied in hypothyroid rodents.

The malformation of the TM in *Thra*<sup>tm1/tm1</sup>*Thrb*<sup>tm1/tm1</sup> mice would impair hair cell mechanosensitive transduction and the tuning of basilar membrane motion, as indicated by other mutations in TM structural components. The complete detachment of the TM in *Tecta*<sup>ΔENT/ΔENT</sup> mice with a large deletion in the entactin-like domain of α-tectorin reduces the sensitivity of basilar membrane motion by 35 dB (Legan et al., 2000). Also, human α-tectorin mutations cause deafness (Verhoeven et al., 1998) and deletions of type XI collagen α2 (McGuire et al., 1999), and otogelin (Simmler et al., 2000) cause TM abnormalities and impair the ABR. The integrity of the compart-



ments formed by the TM, the inner sulcus, and adjacent interdental cells of the spiral limbus may also be critical for auditory function and may contribute to the control of the ionic microenvironment and the potassium recycling that are necessary for hair cell function (Spicer and Schulte, 1998; Steel and Kros, 2001; Ulfendahl et al., 2001). Because the roles of the inner sulcus and its functional relationship to the TM and hair cells are incompletely understood at present, it is possible that other, as yet unknown functions are disrupted by the loss of TRs.

The TM in *Thra<sup>tm1/tm1</sup>Thrb<sup>tm1/tm1</sup>* mice possesses collagen fibrils and is immunoreactive for tectorins and otogelin, suggesting that TRs are not required for expression of these major TM components. A more subtle role for TRs could be in the control of the correct amount and timing of expression of TM components by the greater epithelial ridge (Rau et al., 1999), which could explain the enlargement of the TM in *Thra<sup>tm1/tm1</sup>Thrb<sup>tm1/tm1</sup>* mice. The dysregulated secretion of the TM could also be secondary to the more general delay in the reshaping of the greater epithelial ridge during the delayed differentiation of the inner sulcus. The ultrastructural disarray of the TM in both *Thrb<sup>tm1/tm1</sup>* and *Thra<sup>tm1/tm1</sup>Thrb<sup>tm1/tm1</sup>* mice (Fig. 3) suggests another subtle role, which is at least partly TR $\beta$ -specific, in the formation of the striated sheet matrix of the TM. This could involve, for example, glycosylation or other forms of processing of TM components (Richardson et al., 1987).

This study identifies roles for TRs in the physiological differentiation of both IHCs and OHCs. Immature IHCs and OHCs resemble each other morphologically (Pujol et al., 1997) and functionally (Kros, 1996) before they differentiate into mature hair cell types with distinct properties. In rodent postnatal development, OHCs have been suggested to enter a second phase of differentiation during which they acquire their unique properties including electromotility (Pujol et al., 1997). Although TRs are not required for the commitment to form either IHCs or OHCs, they are required subsequently for the proper maturation of both hair cell types. The defect in OHC nonlinear capacitance in *Thra<sup>tm1/tm1</sup>Thrb<sup>tm1/tm1</sup>* mice is in accord with the altered distortion product otoacoustic emissions, a measure of OHC activity, reported in the *hyt/hyt* hypothyroid mouse strain (Li et al., 1999). It remains to be determined whether the defects in hair cell maturation are because of the absence of TRs within the hair cells or are indirect, perhaps because of abnormalities in maturation factors or in cell–cell interactions that are normally directed by other regions of the organ of Corti.

The stria vascularis has a major role in generating the endocochlear potential in the scala media of the cochlea (Fig. 2*M*), and defects in its function could contribute to the reduced endocochlear potential in *Thra<sup>tm1/tm1</sup>Thrb<sup>tm1/tm1</sup>* mice. This may be consistent with the suggested regulation of NaK-ATPases in the stria vascularis by thyroid hormone (Zuo and Rarey, 1996). It is also possible that this defect originates elsewhere in the cochlea, for example in cells of the spiral limbus that may be involved in potassium recycling (Spicer and Schulte, 1998; Steel and Kros, 2001). The IHC and OHC defects together with the abnormal TM and low endocochlear potential could explain the profound abrogation of auditory function found in *Thra<sup>tm1/tm1</sup>Thrb<sup>tm1/tm1</sup>* mice, as indicated by the severely defective ABR. This need not exclude additional roles for TRs in more central auditory pathways, as is suggested in hypothyroid rodents that show changes in innervation and myelination of the cochlear nerve (Uziel, 1986; Knipper et al., 1998), in expression of type 2 deiodinase in

brainstem cochlear nuclei (Guadaño-Ferraz et al., 1999) and in pyramidal cell morphology in the auditory cortex (Ruiz-Marcos et al., 1983).

Several features of the cochlear phenotype, including the retarded development of the inner sulcus and IHC  $I_{K,f}$  current reflect delays rather than permanent defects. Thus, other signals or transcriptional pathways (Corey and Breakefield, 1994) must set the ultimate developmental course for these events, whereas TRs confer correct timing. Conceivably, the maturation of auditory function may require activity and sensory inflow during critical periods (Rübsamen and Lippe, 1997; Rüsch et al., 1998), perhaps resembling other sensory systems, such as vision (Katz and Shatz, 1996). As ligand-dependent transcription factors, TRs are well adapted to such a role because they can alter the cochlear transcriptional program in response to temporal signals provided by rising thyroid hormone levels in development (Campos-Barros et al., 2000). Thyroid hormone also has a timing role in amphibian metamorphosis (Huang et al., 2001), suggesting that an interplay between rising hormone levels and specific TRs in target tissues provides a timing control that can be adapted to very different processes in vertebrate development.

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